IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Rasmus Dines Larsen et al.
Serial No.: 10/533,324
Filed: August 12, 2005

Examiner: Robert Thomas Crow
Art Unit: 1634
Attorney's docket: HOI-13202/16

Title: METHOD FOR ASSESSMENT OF PARTICLES

DECLARATION by Rolf Henrik Berg, under 37 C.F.R. § 1.132

I, Rolf Henrik Berg, declare and state as follows:

1. I am employed as a full professor in the Department of Micro- and Nanotechnology at the Technical University of Denmark (cf. www.nanotech.dtu.dk/Berg). Also, I am Vice President at ChemoMetec A/S.

   I received my MSc and PhD in chemistry from the University of Copenhagen.

   Honours include elected member of the Danish Academy of Technical Sciences (ATV), elected member of the Danish Academy of Natural Sciences, and Personally Invited Nominator to the Nobel Committee for Chemistry during the last eight years (annual invitations since 2001).

   I have published about 50 scientific articles (including three in *Nature* or *Science* – the two most prestigious scientific journals in the world) which in total have received more than 5,000 citations. I have more than 25 years of research experience in polymer-supported (solid-phase) chemistry, biomolecular chemistry, peptides, nucleic acids, diagnostics (detection) and systems integrating optics/lasers and molecular materials.

   From 1986 to 1989, I was a Research Fellow at Rockefeller University (New York) under the supervision of Bruce Merrifield who (solely) won the 1984 Nobel Prize in Chemistry for developing methodology for chemical synthesis on a solid matrix. Together with Merrifield, I reported a novel concept for immobilization and synthesis of peptides on polymer supports in *Journal of the American Chemical Society* – one of the most prestigious chemistry journal in the world (cf., Berg, Merrifield *et al. J. Am. Chem. Soc. 111,* 8024 (1989)).

   Most notably, I'm co-inventor of peptide nucleic acids (PNA), cf. Nielsen *et al., Science 254,* 1497 (1991). PNA represents the first DNA backbone replacement and its properties and uses are referred to in many scientific articles and several US patents. Other important inventions/ discoveries include the discovery of photoanisotropic peptides (Berg *et al., Nature 365,* 566 (1993)) which was highlighted on *Nature*'s front cover and further highlighted in a News and View

piece in the journal as well as in a Science and Technology piece of *The Economist*. In total, I'm named inventor of 39 issued or pending US patents (and foreign equivalents).
Past and present services include member of the Editorial Advisory Board of the *Journal of Peptide Science*, the Scientific Advisory Board of Santaris Pharma A/S, and Council of the European Peptide Society (representing Denmark) as well as a number of invited submissions to science foundations, ministries and committees including Swiss National Science Foundation (1996-2009) and Japanese Ministry of Trade and Industry (1996-1998).

2. I have studied the above-mentioned application (hereinafter, the invention). In addition, I have reviewed the Office Action of 05 January 2010 and have also evaluated Lea et al. (US 5,428,451), Kaplan et al. (US 6,280,961) and Hansen et al. (EP 1,180,675) for the purpose of assessing at least one quality parameter or at least one quantity parameter of a particle in a liquid material, as described in the invention.

3. I have reviewed and understood the office action of 05 January 2010 regarding above mentioned patent application in view of cited art of Lea et al., Kaplan et al. and Hansen et al.

4. The office action acknowledges that Lea does not disclose all the features of the main claim of the present application. In that regard, the office action does not explicitly teach the amount of analyte detectable position being less than 1×10^6 per particle and it does not show the particle stream being at standstill during the exposure of the signal onto the array of detectors.

5. However, in rejecting the claims of the present application, the office action asserts that: (1) Kaplan teaches a method wherein an analyte of interest that is present at less than 20,000 molecules/ cell (or particles) in one or more cells or particles is detected; (2) Lea teaches adjusting the flow speed of the particle stream used therein to provide for optimum imaging; (3) Kaplan teaches detecting analytes in a cell in the form of a plate and (4) Hansen discloses measurements being carried out on a sample, which is at standstill. Therefore, the office action considers that claims under prosecution for the invention are obvious when the teachings of Lea, Kaplan and Hansen are combined together.

6. The office action mentions that the ordinary artisan would have been motivated to modify Lea with the teachings of Kaplan because the modification would have resulted in a method having the added advantage of allowing detection of analytes at their normal level in the cell, as the detected analytes do not have to be overexpressed in a cell. Similarly, motivation to modify Lea with the teachings of Hansen is provided, such modification would have resulted in a method having an added advantage of improved sensitivity as a result of allowing detection of any weak signals which might indicate the presence of a particle.

7. The present invention relates to a method for the detection and analysis of particles in a liquid material through the use of a reagent which comprises a labelled targeting species. The targeting species is capable of selectively binding to analyte detectable positions on the particle and the labelling agent is a compound capable of emitting, absorbing, attenuating or scattering electromagnetic radiation to result in the generation of a detectable electromagnetic signal. The method is directed to those situations wherein the number of analyte detectable positions on the particles is very low, such as less than 1×10^6 positions per particle. As a consequence, the detectable signal produced by the reagent material is quite low. The low number of analyte detectable positions is attributed to the surface based (non-core) cell binding mechanism. Therefore, in the present invention the admittedly novel combination of optics and experimental conditions used, such as detection when the sample is at standstill, are crucial.

8. The invention aims to solve the technical problem of "How to measure labelled particles with a low amount of a visual label", which is well correlated with the object stated in the description of the present invention on page 2, lines 13-15 stating "It is an object of the present invention to provide an alternative method for the assessment of properties related to particles based on staining of analytes which are several orders of magnitude less abundant than DNA monomers."

9. Prior art methods relying on cell core labelling such as fluorescent labelling of DNA in cellular material typically produce 3×10^9 detectable positions per particle and hence, invariably produce an intense electromagnetic detectable signal. Such intense signals can easily be captured from each cell in a short period of time. Accordingly, in such prior art methods, the analysis can be performed even on a moving particle sample flowing past a detection element, by hydrodynamic techniques such as flow cytometery.

10. The office action refers to col. 4, lines 19-21 in Lea to indicate that Lea characterizes its method as being an alternative to flow cytometers. Whether or not the Lea method is described as flow cytometry is immaterial to the present office action. There is no dispute that the Lea method is carried out on a flowing sample and is inherently a hydrodynamic technique. However, the referred passage of Lea only suggests using a different light source other than laser light and does not characterize the Lea method as differing from that of conventional flow cytometers. Therefore, the office action mistakenly interprets the statement. For purposes of my discussion, I will refer to the Lea process as being hydrodynamic, with the understanding that this term encompasses flow cytometry.

11. Lea discloses a method in which fluorescent particles in a fluid are counted by passing the fluid through an optical cell and an image of said particles is projected onto an array of charge coupled devices (abstract; claim 1; col. 2, lines 19-23). Therefore, the cited art explicitly discloses hydrodynamic flow cytometry technique. In flow cytometry, the sample is transported through an

interrogation point and for accurate detection of signal and data collection from each particle that passed through the interrogation point; it is important that particles or cells are passed through the beam one-at-a-time, i.e. the flow is arranged so that there is a large separation between cells relative to their diameter to avoid any overlap between the particles. This feature of flow cytometry is implemented in the cited method as well (col. 3, line 68 – col. 4, line 2). Reliable signal detection under such hydrodynamic focussing is possible only if a large electromagnetic signal can be captured from each cell in a short period of time. This indicates that the cited art method can hence only be used for assessing parameters of particles having a large number of detectable positions (see item 9 of the declaration), which is only attainable through cell core labelling.

12. In Lea, large separations between the cells/ particles and passage of each particle through the interrogation point one-after-another results in sequential detection of only one particle at a time. In contrast, the method of the present invention requires simultaneous detection of a plurality of particles. Lea method is incapable of performing such simultaneous detection of more than one particle because there is no suggestion made in Lea about the possibility of making such a modification in the cited system of Lea. Moreover, if such modification is incorporated in Lea's system, then the modified system would fail to comply with the working principle of flow cytometry.

13. Furthermore, hydrodynamic method of Lea will introduce error that are inherent in the detection of signals from a moving particle sample, because of variation in flow conditions, particularly when an assessment of a property is a volume related property such as the counting of particles in a volume of sample. Although, Lea mentions that if the particle stream is found to be moving too fast for accurate counting, the speed may be controlled (col. 3, lines 20-30), but the Lea method relies on being able to rapidly examine and distinguish between different particles in a liquid flow. In Lea and all other hydrodynamic techniques, the speed control only ensures that there is a large separation between consecutive particles, thereby avoiding any overlap. A skilled person employing hydrodynamic techniques such as that of the Lea method is thus looking for a high throughput screening of particles to obtain a detectable signal. Therefore, even if the speed is reduced, no skilled person, while using the Lea technique would make the sample to stand still because that would disrupt the working principle of Lea as it is based on hydrodynamic scanning of one particle at a time. Hence, standstill sample assessment is contrary to the scope of the Lea technique. More specifically, the development work in hydrodynamic techniques such as flow cytometry, is directed towards increasing the flow speed of the particles and scanning reliably the flowing particles individually, rather than assessing multiple particles at a time by bringing the speed down to standstill. Therefore, any skilled person using the teaching of Lea et al. would try the taught method only with particles having a high number of detectable positions because this enables detection of signals from particles under flow condition.

14. Because in hydrodynamic processes such as that of Lea, detection of signals from each labelled particle is performed individually and one-at-a-time, therefore, bringing the speed of the particle to zero and incorporating a thin flow path (col. 3, line 68 – col. 4, line 2), as suggested by the Office Action would at the best result in illumination of only one particle, the one which is stopped at the detection point. Consequently, the combination suggested by the office action will not be able to illuminate and detect a plurality of the particles in the sample simultaneously as is the case in the present invention. Moreover, as explained under item 13, bringing the speed to zero is not in compliance with the teachings of Lea and this item therefore, only refers to a hypothetical situation for which there is no teaching in Lea and detection under this situation is ineffective and of no practical use. Furthermore, repetitive process of stopping and starting fluid flow in flow cytometry would invariably result in mixing up of the labeled particles, hence, contravening the principle of keeping large separation between the particles, as required by hydrodynamic techniques such as that of Lea.

15. The claims of the present invention differ from the prior art, because the claimed method is not a hydrodynamic method and does not take measurements on a flowing sample. Instead, it employs an imaging system that can image cells that are at a standstill position, i.e. it is a hydrostatic method, thereby enabling hydrostatic focussing and reading of the signal from more than one particle at any given time. This is advantageous over the hydrodynamic method of Lea because it allows for specified detected events to be revisited, after initial measurements, for a more extensive analysis. This also allows optimal use of measurement time in order to improve any signal to noise conditions and also to detect weak signals to assess particles with low detectable positions. This is certainly not the case with Lea et al.'s work.

16. The office action co-relates blood cells of Lea et al. with preferred form of particles, i.e. blood cells of the invention, and concludes that Lea's reference teaches assessing particles having less than $1X10^6$ detectable positions. However, there is neither any reference in the cited art that the detectable positions in the blood cell are less than $1 X 10^6$ nor any teaching for such low number of detectable positions. Furthermore, it is to be noted that DNA (cell core) binding produces a much high number of (approximately $3 x 10^9$) detectable positions for the same blood cell as opposed to that obtained from surface binding, which will produce less than $1X10^6$ detectable positions. Therefore, use of the same cell is certainly not an indication of available active detectable positions. Lea's hydrodynamic method will work well with blood cells having DNA (cell core) binding because this produces detectable signal under particle flow condition because of a large number of detectable positions. However, such flow assessment method will not work reliably with blood cells having surface binding reagents.

17. The office action further points out that the particles are bound to reagents in the form of superparamanetic beads or via a sandwich complex (col. 4, lines 25-col. 5, line 5). However, the cited art further mentions that the magnetic particles are typically attached to selected cells,

which after processing, may be more convenient to lyse and only the nuclei which have previously been stained by a fluorescent dye are counted (col. 4, lines 59-62). Use of "may" in the previous statement refers to the possibility of higher convenience in lysing a cell after the processing and not whether the cells are lysed. In other words, the cells are necessarily lysed. This clearly indicates that before the use of the hydrodynamic technique, the Lea method requires breaking the cellular membrane and creating a lysate, followed by counting only the stained nuclei (cell core), thereby indicating production of high number of detectable positions, as described above, and hence making the cited art principally different from the present invention.

18. The method of the invention is carried out at a low magnification (smaller than 20:1), whereby it is possible to detect all particles in a large volume in one or a few exposures. The low magnification offer several advantages, such as increased area of observation and increased depth of focusing, implying increased volume exposed to the detection device (Page 23, lines 3-34). Although, Lea mentions that the area of the image of each particle at the said array is approximately the same as the area of at least a single charge coupled device, indicating a 1:1 relation but the cited art neither recognizes the magnification as a variable that would achieve the recognized result of simultaneously detecting signals from a plurality of weakly labelled particles (see, *In re Antonie*, 559 F.2d 618, 195 USPQ 6 (CCPA 1977)). Therefore, indication of magnification 1:1 relation *per se* in Lea would not suggest to a person skilled in the art to modify the magnification to less than 20:1.

19. There is no evidence or suggestion in Lea reference relating to the technical problem that is solved by the invention, thereby lacking enough teaching to suggest a skilled person to relate Lea reference with proposed solution of the present invention. Consequently, from Lea's teachings, a skilled person is restricted only to a hydrodynamic technique and with particles having a high number of detectable positions. In fact, the fundamental difference, among others, between the Lea and present invention is the technical problem that a skilled person will face. Because, Lea's work operates in a hydrodynamic area and does not touch upon the issue of particles having low amount of visual label, there is no probability that a skilled person will look and interpret the teachings of Lea to solve the technical problem.

20. Even if, a person skilled in the art with application of common sense attempts to employ the teachings of Lea, he will have to think and work in lines of standstill (hydrostatic) positioning of the sample to solve the technical problem. This is a non-obvious and conceptually divergent technique from Lea's hydrodynamic technique. There is also no hint provided to consider using the method of Lea in the special case of assessing qualitative and other quantitative parameters of particles, on which labelling agents bind to analytes present only in a low number on each particle.

21. I conclude that Lea is not a relevant art for combination with any other art because of fundamentally opposite scope of the cited art in comparison to the present invention.

22. Hansen discloses a method and system for assessing properties of particles in a liquid sample. The assessment is performed by staining the particles with stains which are known to stain DNA monomers (about 3 x 10^9 available detectable positions). Accordingly, Hansen discloses methods for assessment based on staining abundant molecules in the cells. Under such conditions, the signal accumulated from each cell is relatively high and the signal to noise ratio is correspondingly low so that distinction between signal from particles and background is facilitated. Although, Hansen teaches assessment of particles under stand still positions (Para 70), but there is no teaching in Hansen to solve the technical problem of "How to measure labelled particles with a low amount of a visual label" less than $1X10^6$.

23. In combination with Lea, it is noted that Lea teaches assessing one particle at a time by employing hydrodynamic, flowing sample technique such as flow cytometry, whereas Hansen primarily assesses stand still particles. The principles employed in Lea and Hansen are contradictory in nature, and if combined i.e. if sample speed in flow cytometer is brought down to zero, then Lea's teaching would seize to operate therefore disrupting the basic teaching and working principle of Lea. The only common feature between these two prior art references is their incapability to assess particles having low number of detectable positions.

24. The teachings of Lea and Hansen are not just different but contradictory in nature and, therefore, a skilled person would not consider combining the teachings of these cited arts. An artisan at the time of the invention would not have reasonably considered embedding a hydrostatic teaching of Hansen within an explicitly taught hydrodynamic teaching of Lea in the manner suggested in the Office Action.

25. Kaplan discloses a system for analyzing cellular materials wherein the density of analytical sites on the surface of the cell is low, and in this regard less that 1 X 10^6 per particle. Kaplan mentions that hard to detect analytes may be readily detected by the increase in the labeling of the cell or particle caused by the amplification of the labelling molecule (col. 2, lines 17-21). This is further exemplified at col. 7, lines 12-43 where cells or particles are tyramide coated for physical separation using serial amplification. Therefore, Kaplan is not relevant to the teachings of the invention because of the low density of particles; an analyte dependent enzyme activation system (ADEAS) must be used to provide an enhanced signal. As such, Kaplan teaches away from the principles of the present invention insofar it suggests to one of the skilled in the art that the chemical ADEAS amplification system must be employed in analyses of this type.

26. In the present invention, the labelling agent is a compound that is capable of emitting, absorbing, attenuating or scattering electromagnetic radiation to result in the generation of a detectable

electromagnetic signal and the labelling agent selectively and directly binds to an analyte position. However, in Kaplan, detectable markers are added after tyramide coating to facilitate physical separation. Tyramide coating over the surface of cells or particles involves catalytic activity of the enzymatic portion of a binding partner and an appropriate substrate resulting in inducing chemical changes in other substances. To be able to detect and physically separate labelled particles, Kaplan utilizes a method for tyramide coating cells or particles using serial amplification, therefore, principle of labelling of particles/ cells in Kaplan is different from that in the present invention and incorporating teachings of Kaplan in that of Lea makes it different from claims of the present invention.

27. The office action states that Kaplan's use of plates and dishes suggests using standstill sample. However, the reasons included under items 23 and 24 are applicable to the combined teachings of Lea and Kaplan, where it is emphasized that any attempt to incorporate standstill sample in the hydrodynamic flow cytometry mechanism of Lea is in violation to the principle of operation of Lea's method. Therefore, any conclusion suggesting such a combined system as workable is unreasonable.

28. Difference in working principles of Kaplan and Lea is further illustrated in Kaplan through its various examples where washing steps are used. The washing of the plate indicates that the labelled particles are supported on a solid support, and are therefore, immobilized. This allows the particles to withstand the washing. However, such washing steps are incompatible with the hydrodynamic method of Lea because the particles are not immobilized on any solid support. In fact, the sample particles in Lea are in constant continuous movement through the optical cell. Therefore, incorporating teachings of Kaplan in Lea makes Lea's method unworkable.

29. I, therefore, conclude that combination of the hydrodynamic, flowing sample method of Lea and standstill assessment method of Hansen is not workable. The working principle of Kaplan is different from that of the present invention because the cited art makes the use of amplification of the labelling molecule as a mandatory requirement. Kaplan is inconsequential to determine inventiveness of the present invention because it fails to correctly disclose at least the labelling mechanism of the present invention. Therefore, combining Kaplan's teaching to that of Lea's would result in a combined system, which is principally, different from the one claimed in the present invention. As such, combining teachings of these three cited arts is not only improper and driven by impermissible hindsight but also inconclusive and confusing because of the differences in the operating principles of these arts.

30. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 16 April 2010

Signature: _____

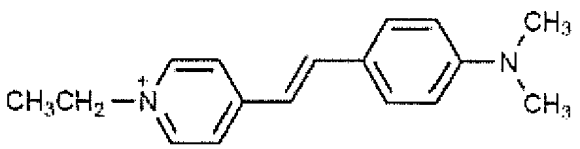
(Rolf Henrik Berg)

EXHIBIT 1

Cyanine

From Wikipedia, the free encyclopedia

Cyanine is a non-systematic name of a synthetic dye family belonging to polymethine group. Cyanines have many uses as fluorescent dyes, particularly in biomedical imaging. Depending on the structure, they cover the spectrum from IR to UV.



Cyanines were originally used, and still are, to increase the sensitivity range of photographic emulsions, i.e., to increase the range of wavelengths which will form an image on the film. Cyanines are also used in CD-R and DVD-R media. The ones used are mostly green or light blue in color, and are chemically unstable. This makes cyanine discs unsuitable for archival CD and DVD use, as they can fade and become unreadable in a few years, however, recent cyanine discs contain stabilizers that slow down the deterioration significantly.

Cyanines were first synthesized over a century ago, and there are a large number reported in the literature.

Contents

Structure

There are three types of cyanines:

- *Streptocyanines* or *open chain cyanines*:

  $R_2N^+=CH[CH=CH]_n\text{-}NR_2$ **(I)**

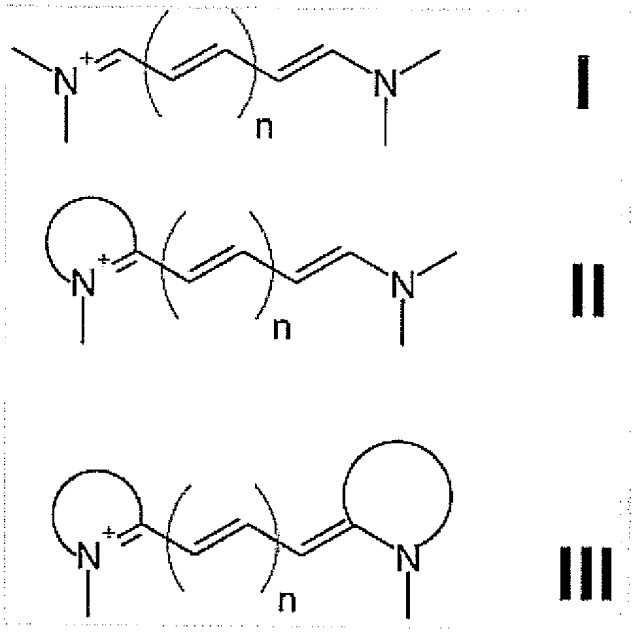
- *Hemicyanines*:

  $Aryl=N^+=CH[CH=CH]_n\text{-}NR_2$ **(II)**

- *Closed chain cyanines*:[1]

Aryl=N^+=CH[CH=CH]$_n$-N=Aryl **(III)**

where two nitrogens are joined by a polymethine chain.[2] Both nitrogens are each independently part of a heteroaromatic moiety, such as pyrrole, imidazole, thiazole, pyridine, quinoline, indole, benzothiazole, etc.



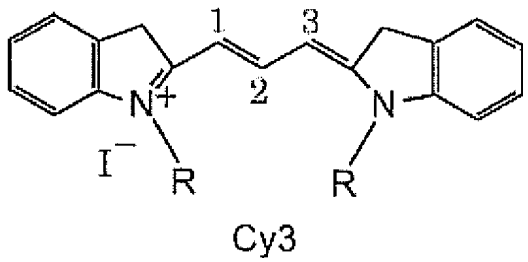
The Cyanines:
I = Streptocyanines,
II = Hemicyanines,
III = Closed cyanine

Cy3 and Cy5

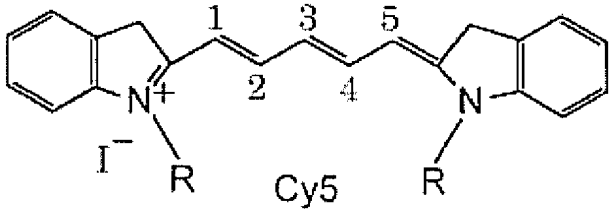
Cy3 and Cy5 are reactive water-soluble fluorescent dyes of the cyanine dye family. Cy3 dyes are red (~550 nm excitation, ~570 nm emission and therefore appear green), while Cy5 is fluorescent in the red region (~650/670 nm) but absorbs in the orange region (~649 nm)[3] .[4] They are usually synthesized with reactive groups on either one or both of the nitrogen side chains so that they can be chemically linked to either nucleic acids or protein molecules. Labeling is done for visualization and quantification purposes. They are used in a wide variety of biological applications including comparative genomic hybridization and in gene chips, which are used in transcriptomics. They are also used to label proteins and nucleic acid for various studies including proteomics and RNA localization.[5]

Nomenclature and Structure

Standard chemical names specify exactly the chemical structure of the molecule. The Cy3 and Cy5 nomenclature was first proposed by Ernst, et al.[2] in 1989, and is non-standard, since it gives no hint of their chemical structures. In the original paper the number designated the count of the methines (as shown), and the side chains were unspecified. Thus various structures are designated Cy3 and Cy5 in the literature.



Cy3



Cy5

The R groups do not have to be identical. In the dyes as used they are short aliphatic chains one or both of which ends in a highly reactive moieties such as N-hydroxysuccinimide or maleimide.

Spectral characteristics

Cy3 is excited maximally at 550 nm and emits maximally at 570 nm, in the orange-red part of the spectrum; quantum yield in PBS buffer is 0.04[6]; FW=766.

Cy5 is excited maximally at 649 nm and emits maximally at 670 nm, in the far red part of the spectrum;

quantum yield is 0.28. FW=792.

The scanners actually use different laser emission wavelengths (typically 532 nm and 635 nm) and filter wavelengths (550-600 nm and 655-695 nm) to avoid background contamination. They are thus able to easily distinguish between two samples when one sample has been labeled with Cy3 and the other labeled with Cy5. They are also able to quantitate the amount of labeling in either sample.

Cy dye alternatives

Alexa Fluor dyes, Dylight, IRIS Dyes, Seta dyes, SeTau dyes, SRfluor dyes and Square dyes dyes can be used interchangeably with Cy dyes in most biochemical applications.

Cy5 ozone susceptibility

In 2003, researchers at Inpharmatics and Agilent reported in *Analytical Chemistry* that microarrays which used Cy5 were susceptible to intermittent data quality decrease caused by environmental ozone. Exposures to ozone levels above 5-10 ppb for 10–30 seconds were reported to decrease the reproducibility of Cy5 microarrays. Much higher levels of ozone (>100 ppb) were required to observe an effect in Cy3 [7].

Applications

Nucleic acid labeling

In microarray experiments DNA or RNA is labeled with either Cy3 or Cy5 that has been synthesized to carry an N-hydroxysuccinimidyl ester (NHS-ester) reactive group. Since, NHS-esters react readily only with aliphatic amine groups, which nucleic acids lack, nucleotides have to be modified with aminoallyl groups. This is done through incorporating aminoallyl-modified nucleotides during synthesis reactions. A good ratio is a label every 60 bases such that the labels are not too close to each other, thus resulting in quenching effects.

Protein labeling

For protein labeling, Cy3 and Cy5 dyes sometimes bear maleimide reactive groups instead. The maleimide functionality allows conjugation of the fluorescent dye to the sulfhydryl group of cysteine residues. Cysteines can be added and removed from the protein domain of interest via PCR mutagenesis.

Cy5, is sensitive to the electronic environment it resides in. Changes in the conformation of the protein it is attached to will produce an enhancement or quenching of the emission. The rate of this change can be measured to determine enzyme kinetic parameters. The dyes can be used for similar purposes in FRET experiments.

Cy3 and Cy5 are used in proteomics experiments so that samples from two sources can be mixed and run together thorough the separation process[8]. This eliminates variations due to differing experimental conditions that are inevitable if the samples were run separately. These variations make it extremely difficult, if not impossible, to use computers to automate the acquisition of the data after the separation is complete. Using these dyes makes the automation trivial.

See also

- Merocyanine
- N-Hydroxysuccinimide
- Maleimide
- Indocyanine green

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